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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Denis-Claude ROY, et al.
SERIAL NO.: NEW U.S. PCT APPLICATION
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INTERNATIONAL APPLICATION NO.: PCT/CA00/01142
INTERNATIONAL FILING DATE: October 3, 2000
FOR: RHODAMINE DERIVATIVES FOR PHOTODYNAMIC DIAGNOSIS AND TREATMENT

**REQUEST FOR PRIORITY UNDER 35 U.S.C. 119(e)
AND THE INTERNATIONAL CONVENTION**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In the matter of the above-identified application for patent, notice is hereby given that the applicant claims as priority:

COUNTRY	APPLICATION NO	DAY/MONTH/YEAR
USA	60/157,790	05 October 1999

Certified copies of the corresponding Convention application(s) were submitted to the International Bureau in PCT Application No. PCT/CA00/01142.

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October 12, 2000

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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	12411-2J "USPR" FC/ld	Type a plus sign (+) inside this box→	+

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TITLE OF THE INVENTION *(280 characters max)*

RHODAMINE DERIVATIVES FOR PHOTODYNAMIC PREVENTION AND TREATMENT OF IMMUNOREACTIVE DISORDERS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

X	Specification	*Number of Pages*	38		Small Entity Statement	
X	Drawings	*Number of Sheets*	8		Other (specify)	

METHOD OF PAYMENT *(check one)*

	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT ($)	$150.00
X	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 19-5113		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government

[X] No

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE: France Côté Date: 10/051999

TYPED or PRINTED NAME: France Côté REGISTRATION NO. *(if appropriate)*: 37,037

[ ] Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

RHODAMINE DERIVATIVES FOR PHOTODYNAMIC PREVENTION AND TREATMENT OF IMMUNOREACTIVE DISORDERS

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a photodynamic treatment for the selective destruction immunologically reactive cells without affecting the normal cells and without causing systemic toxicity for the patient.

(b) Description of Prior Art

Immunologic disorders are uncontrolled cell proliferations that result from the production of immune cells recognizing normal cells and tissues as foreign. After a variable latency period during which they are clinically silent, cells with immunoreactivity towards normal cells induce damages in these normal cells and tissues. Such immunologic disorders are usually divided in alloimmune conditions and autoimmune conditions. Alloimmune disorders occur primarily in the context of allogeneic transplantation (bone marrow and other organs: kidney, heart, liver, lung, etc.). In the setting of bone marrow transplantation, donor immune cells present in the hematopoietic stem cell graft react towards host normal tissues, causing graft-versus-host disease (GVHD). The GVHD induces damage primarily to the liver, skin, colon, lung, eyes and mouth. Autoimmune disorders are comprised of a number of arthritic conditions, such as rhumatoid arthritis, scleroderma and lupus erythematosus; endocrine conditions, such as diabetes mellitus; neurologic conditions, such as multiple sclerosis and myasthenia gravis; anemia, etc. The immune reaction in both alloimmune

and autoimmune disorders progresses to generate organ dysfunction and damage.

Despite important advances in treatment, immunologic complications remain the primary cause of failure of allogeneic transplantations, whether in hematopoietic stem cell transplantation (GVHD) or in solid organ transplantation (graft rejection). In addition, autoimmune disorders represent a major cause of both morbidity and mortality. Prevention and treatment of these immune disorders has relied mainly on the use of immunosuppressive agents, monoclonal antibody-based therapies, radiation therapy, and more recently molecular inhibitors. Significant improvement in outcome has occured with the continued development of combined modalities, but for a small number of disorders and patients. However, for the most frequent types of transplantations (bone marrow, kidney, liver, heart and lung), and for most immune disorders (rhumatoid arthritis, connective tissue diseases, multiple sclerosis, etc.) resolution of the immunologic dysfunction and cure has not been achieved. Therefore, the development of new approaches for the prevention and treatment of patients with immunologic disorders is critically neeeded particularly for those patients who are at high risk or whose disease has progressed and are refractory to standard immunosuppressive therapy. Allogeneic stem cell transplantation (AlloSCT) has been employed for the treatment of a number of malignant and non-malignant conditions. Allogeneic stem cell transplantation is based on the administration of high-dose chemotherapy with or without total body irradiation to eliminate malignant cells, and host hematopoietic cells. Normal hematopoietic donor stem cells are then infused into

the patient in order to replace the host hematopoietic system. AlloSCT has been shown to induce increased response rates when compared with standard therapeutic options. One important issue that needs to be stressed when using AlloSCT relates to the risk of reinfusing immune cells that will subsequently recognize patient cells as foreign and cause GVHD. A variety of techniques have been developed that can deplete up to 10^5 of T cells from the marrow or peripheral blood. These techniques, including immunologic and pharmacologic purging, are not entirely satisfactory. One major consideration when purging stem cell grafts is to preserve the non-hostreactive T cells so that they can exert anti-infectious and anti-leukemia activity upon grafting. The potential of photodynamic therapy, in association with photosensitizing molecules capable of destroying immunologically reactive cells while sparing normal host-non-reactive immune cells, to purge hematopoietic cell grafts in preparation for AlloSCT or autologous stem cell transplantation (AutoSct), and after AllcSCT in the context of donor lymphocyte infusions to eliminate recurring leukemia cells has largely been unexplored. To achieve eradication of T cells, several approaches have been proposed including:

1) *in vitro* exposure of the graft to monoclonal antibodies and immunotoxins against antigens present on the surface of T cells (anti-CD3, anti-CD6, anti-CD8, etc.);
2) *in vitro* selection by soybean agglutinin and sheep red blood cell rosetting;
3) positive selection of CD34+ stem cells; and
4) *in vivo* therapy with combinations of anti-thymocyte globulin, or monoclonal antibodies.

5) *In vitro* exposure of recipient-reactive donor T cells by monoclonal antibodies or immunotoxins targeting the interleukin 2 receptor or OX-40 antigen (Cavazzana-Calvo M. et al. (1990) Transplantation, 50:1-7; Tittle T.V. et al (1997) Blood 89:4652-58; Harris D.T. et al. (1999) Bone Marrow Transplantation 23:137-44)

However, most of these methods are not specifically directed at the alloreactive T cell subset and associated with numerous problems, including disease recurrence, graft rejection, second malignancies and severe infections. In addition, the clinical relevance of several of these methods remains to be established.

There are many reports on the use of photodynamic therapy in the treatment of malignancies (Daniell M. D., Hill J. S. (1991) *Aust. N. Z. J. Surg.*, **61**: 340-348). The method has been applied for cancers of various origins and more recently for the eradication of viruses and pathogens (Raab O. (1900) *Infusoria Z. Biol.*, **39**: 524).

The initial experiments on the use photodynamic therapy for cancer treatment using various naturally occuring or synthetically produced photoactivable substances were published early this century (Jesionek A., Tappeiner V.H. (1903) *Muench Med Wochneshr*, 47: 2042; Hausman W. (1911) *Biochem. Z.*, **30**: 276). In the 40's and 60's, a variety of tumor types were subjected to photodynamic therapy both *in vitro* and *in vivo* (Kessel, David (1990) *Photodynamic Therapy of neoplastic disease*, Vol. I, II, CRC Press. David Kessel, Ed. ISBN 0-8493-5816-7 (v. 1), ISBN 0-8493-5817-5 (v. 2)). Dougherty et al. and others, in the 70's and 80's, systematically explored the potential of oncologic application of photodynamic therapy

(Dougherty T. J. (1974) *J. Natl Cancer Inst.*, **51**: 1333-1336; Dougherty T. J. et al. (1975) *J. Natl Cancer Inst.*, **55**: 115-121; Dougherty T. J. et al. (1978) *Cancer Res.*, **38**: 2628-2635; Dougherty T. J. (1984) *Urol. Suppl.*, **23**: 61; Dougherty T. J. (1987) *Photochem. Photobiol.*, **45**: 874-889).

Treatment of immunoreactive cells with photodynamic therapy

There is currently a lack of agents which allow selective destruction of immunoreactive cells while leaving intact the normal but suppressed residual cellular population. Preferential uptake of photosensitive dye and cytotoxicity of photodynamic therapy against leukemia (Jamieson C. H. et al. (1990) *Leuk. Res.*, 14: 209-219) and lymphoid (Greinix H.T., et al. Blood (1998) 92:3098-3104; and reviewed in Zic J.A. et al. Therapeutic Apheresis (1999) 3:50-62) cells have been previously demonstrated.

It would be highly desirable to be provided with photosensitizers which possess the following characteristics:

i) preferential localization and uptake by the immunoreactive cells;

ii) upon application of appropriate light intensities, killing those cells which have accumulated and retained the photosensiting agents;

iii) sparing of the normal hemopoietic stem cell compartment from the destructive effects of activated photosensitizers; and

iv) potential utilization of photosensitizers for hematopoietic stem cell purging of immunoreactive cells in preparation for allogeneic or autologous stem cell transplantation.

v) Potential utilization of photosensitizers for *ex vivo* elimination of reactive immune cells in patients with immunological disorders.

The Rhodamine dyes

Rhodamine 123 (2-(6-amino-3-imino-3H-xanthen-9-yl) benzoic acid methyl ester hydrochloride), a lipophilic cationic dye of the pyrylium class which can disrupt cellular homeostasis and be cytostatic or cytotoxic upon high concentration exposure and/or photodynamic therapy, although with a very poor quantum yield (Darzynkiewicz Z., Carter S. (1988) *Cancer Res.*, 48: 1295-1299). It has been used *in vitro* as a specific fluorescent stain for living mitochondria. It is taken up and is preferentially retained by many tumor cell types, impairing their proliferation and survival by altering membrane and mitochondrial function (Oseroff A. R. (1992) In *Photodynamic therapy* (Henderson B. W., Dougherty T. J., eds) New York: Marcel Dekker, pp. 79-91). In vivo, chemotherapy with rhodamine 123 can prolong the survival of cancerous mice, but, despite initial attemps to utilize rhodamine 123 in the treatment of tumors, its systemic toxicity of rhodamine 123 may limit its usefulness (Bernal,S.D., et al. (1983) *Science*, 222: 169; Powers,S.K. et al. (1987) *J. Neurosur.*, 67: 889).

United States Patent No. 4,612,007 issued on September 16, 1986 in the name of Richard L. Edelson, discloses a method for externally treating human blood, with the objective of reducing the functioning lymphocyte population in the blood system of a human subject. The blood, withdrawn from the subject, is passed through an ultraviolet radiation field in the presence of a dissolved photoactive agent capable of forming photoadducts with lymphocytic-DNA. This

method presents the following disadvantages and deficiencies. The procedure described is based on the utilization of known commercially available photoactive chemical agents for externally treating patient's blood, leaving the bone marrow and potential resident leukemic clones intact in the process. According to Richard L. Edelson, the method only reduces, does not eradicate, the target cell population. Moreover, the wavelength range of UV radiation used in the process proposed by Richard L. Edelson could be damageable to the normal cells.

International Application published on January 7, 1993 under International publication number WO 93/00005, discloses a method for inactivating pathogens in a body fluid while minimizing the adverse effects caused by the photosensitive agents. This method essentially consists of treating the cells in the presence of a photoactive agent under conditions that effect the destruction of the pathogen, and of preventing the treated cells from contacting additional extracellular protein for a predetermined period of time. This method is concerned with the eradication of infectious agents from collected blood and its components, prior to storage or transfusion, and does not impede on the present invention.

It would be highly desirable to be provided with a new approach for the use of rhodamine derivatives in the treatment of immunereactive cells which overcomes these drawbacks while having no systemic toxicity for the patient.

SUMMARY OF THE INVENTION

One aim of the present invention is to produce new photosensitizers endowed with the following characteristics:

i) preferential localization and uptake by the immunoreactive cells;

ii) upon application of appropriate light intensities, killing those cells which have accumulated and retained the photosensiting agents;

iii) sparing of the normal hemopoietic stem cell compartment from the destructive effects of activated photosensitizers;

iv) potential utilization of photosensitizers for hematopoietic stem cell purging of immunoreactive cells in preparation for allogeneic or autologous stem cell transplantation; and

v) Potential utilization of photosensatizers for ex vivo elimination of reactive immune cells in patients with immunological disorders.

In accordance with the present invention, there is provided a photoactivable pharmaceutical composition for the selective destruction and/or inactivation of immunologically reactive cells without affecting the normal cells and without causing systemic toxicity for the patient, said composition comprising at least one photoactivable rhodamine derivative selected from the group consisting of 4,5-dibromorhodamine 123 (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid methyl ester hydrochloride); 4, 5-dibromorhodamine 123 (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid ethyl ester hydrochloride); 4, 5-dibromorhodamine 123 (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-

benzoic acid octhyl ester hydrochloride); 4,5-dibromorhodamine 110 n-butyl ester (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid n-butyl ester hydrochloride); Rhodamine B n-butyl ester (2-(6-ethyl amino-3-ethyl imino-3H-xanthen-9-yl)-benzoic acid n-butyl ester hydrochloride); and photoactivable derivatives thereof; in association with a pharmaceutically acceptable carrier; whereby photoactivation of said derivatives induces cell killing while unactivated derivatives are substantially non-toxic to cells.

In accordance with the present invention, there is provided with the use of the photoactivable derivatives for the photodynamic treatment for the selective destruction and/or inactivation of immunologically reactive cells without affecting the normal cells and without causing systemic toxicity for the patient, wherein appropriate intracellular levels of said derivatives are achieved and irradiation of a suitable wavelength and intensity is applied.

In accordance with the present invention, there is provided a method of prevention of graft-versus-host disease associated with allogeneic stem cell transplantation in a patient, which comprises the steps of:

a) activating lymphocytes from a donor by mixing donor cells with host cells for a time sufficient for a period of time sufficient for an immune reaction to occur;

b) substantially eliminating the activated lymphocytes of step a) with photodynamic therapy using a therapeutic amount of a photoactivable composition of claim 1 under irradiation of a suitable wavelength; and

c) performing allogenic stem cell transplantation using the treated mix of step b).

In accordance with the present invention, there is provided a method for the treatment of immunologic disorder in a patient, which comprises the steps of:

a) harvesting said patient's hematopoietic cells;

b) *ex vivo* treating of the hematopoietic cells of step a) by photodynamic therapy using a therapeutic amount of a photoactivable composition of claim 1 under irradiation of a suitable wavelength; and

c) performing graft infusion or autograft transplantation using the treated hematopoietic cells of step b).

The immunologic disorder may be selected from the group consisting of conditions in which self cells or donor cells react against host tissues or foreign targets, such as garft-versus-host disease, graft rejection, autoimmune disorders and T-cell mediated immunoallergies.

The hematopoietic cells may be selected from the group consisting of bone marrow, peripheral blood, and cord blood mononuclear cells.

For the purpose of the present invention the following terms are defined below.

The term "immunoreactive disorders" is intended to mean any alloimmune or autoimmune reaction and/or disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph of the photo toxicity of 4,5-dibromorhodamine 123 (TH9402) used in accordance with the method of the present invention against K562 and CEM cell lines admixed with normal irradiated PBMC and

expressed as a fraction of the number of clonogenic cells;

Fig. 2 demonstrates that PHA activated lymphocytes stop incorporating 3H-thymidine after photodynamic therapy with 7.5 and 5 joules/cm^2, in contrast to medium treated cells.

Fig. 3 demonstrates that cells from subject A activated against B cells and photodynamically treated, do not proliferate when reexposed to B cells but do proliferate when exposed to C cells. A, B and C cells were from unrelated individuals: A and B differed by 2 HLA antigens (B and DR).

Fig.4 shows TH9402 fluorescence upon flow cytometric evaluation of resting and activated lymphocytes. Cells were evaluated at various times after the end of the TH9402 incorporation period. Activated lymphocytes retain more TH9402 than resting lymphocytes.

Fig. 5 shows the impact of cyclosporin A on the TH9402 cellular efflux after 110 minutes from the end of the TH9402 incorporation period. Cyclosporin A blocks the efflux of TH9402 in resting lymphocytes, but not in activated lymphocytes.

Figs. 6A, 6B, and 6C show three graphs of the photo toxicity of 4,5-dibromorhodamine 110 n-butyl ester used in accordance with the method of the present invention and expressed in % viability; and

Figs. 7A and 7B show two graphs of the photo toxicity of rhodamine B n-butyl ester used in accordance with the method of the present invention and expressed in % viability.

DETAILED DESCRIPTION OF THE INVENTION

Photoactive dyes are excited from the ground state to the singlet excited state following

absorption of photons. Singlet excited states of organic molecules generally have short lifetimes (10^{-12}-10^{-6} sec.) as they rapidly relax back to the ground state using non-radiative (vibrational modes) and radiative (fluorescence) processes. Intersystem crossing to the more stable triplet excited state is also competing with relaxation to the ground state. Triplet excited states generally have longer lifetimes (10-6-10 sec) which allow them to diffuse and react with other molecules in the medium.

Reactivity between molecular oxygen and a photosensitizer excited to the triplet state, both present in malignant cells, is the operating principle of most photodynamic therapies. Triplet excited states can react with molecular oxygen via two different mechanisms. The first mechanism (Type I) consists of the transfer of an electron from the excited dyes to molecular oxygen, resulting in highly reactive free radicals being present in the cellular environment.

The second mechanism (Type II) consists of the transfer of energy from the excited dyes to molecular oxygen, leading to the formation of cytotoxic singlet oxygen.

Photosensitizers must therefore meet two conditions in order to be an effective phototherapeutic agent. The first condition is that they must be present at a far higher concentration in target cells than that in normal cells. A higher concentration of dyes in malignant and immunoreactive cells results in a higher concentration of photogenerated cytotoxic species and therefore in a higher death rate. The second condition is that irradiation of the phototherapeutic agent, in the presence of intracellular concentrations of molecular

oxygen, must lead to the formation of the cytotoxic species with high efficiency.

Rhodamine 123 is known to be taken up and preferentially retained by many tumor cells and consequently its use as a phototherapeutic agent has been proposed. However, the singlet excited state of Rhodamine 123 does not undergo intersystem crossing to the triplet excited state efficiently. Because of this, Rhodamine 123 is a weak phototoxin (Morliere, P et al. (1990) *Photochemistry and Photobiology*, 52(4): 703-710).

To overcome the limitations of the prior art methods, the chemical structure of rhodamine 123 can be modified in such a way as to enhance intersystem crossing to the triplet excited state. Theoretically, this could be achieved by substituting heavy atoms, such as Br or other halides, for hydrogen atoms in the molecular structure of rhodamine 123. Therefore, dibromorhodamine 123 (referred herein as TH9402) has been prepared and tested.

The amphiphatic structure and hydrophilicity of the dyes could modulate the cytoplasmic and mitochondrial membranes and affect the phototoxicity of the dye. For example, hydrophobicity was shown to be the most important factor influencing the *in vitro* uptake of porphyrins (Chi-Wei Lin (1990) *In Photodynamic therapy of neoplastic disease*, Vol II, CRC Press, pp 79-101). Therefore, different esters of rhodamine 123 and rhodamine B were prepared and tested. More specifically dibromorhodamine n-butyl ester (DBBE) and rhodamine B n-butyl-ester (RBBE).

Different heavy atom substitutions of the hydrogen atoms (halogenic substitution) of the rhodamine backbone, for example, dibromo and diiodo

derivatives of rhodamine B and Rh 110, are being prepared and tested.

Dimers/oligomers, hetero dimers/oligomers of such compounds also will be prepared and tested.

Substitution of the oxygen heteroatom of the rhodamine backbone by a heavier atom to reduce S_0/S_1 splitting, theoretically should increase spin orbit coupling and promote intersystem crossing from the S_1 to the T_1 state, producing higher triplets yields than the original dye. This should increase proportionally the production of singlet oxygen. Therefore, S (Sulfur), Se (Selenium) and Te (Tellurium) substitutions for the oxygen atom (O) of the rhodamine backbone is explored. More over, other strategies for increasing high quantum yields of Type I (free radicals) or Type II (superoxyde anion or singlet oxygen) products and tumor selective accumulation of the dye are tested.

In accordance with the present invention, there is also shown that TH9402 is preferentially retained by activated T cells.

In accordance with the present invention, there is provided the use of such above-mentioned dyes in conjugation with tumor specific antibodies, or poisonous substances, or liposomal or lipoproteins, or fluorochrome adducts or other agents.

In addition, the photosensitizers to be described have the potential to act synergistically in conjunction with other photoactive substances.

Moreover, the negative selection procedure provided by the use of photodynamic treatment does not preclude the use of other means for enriching hematopoietic stem cells such as positive selection with anti-CD34 monoclonal antibodies.

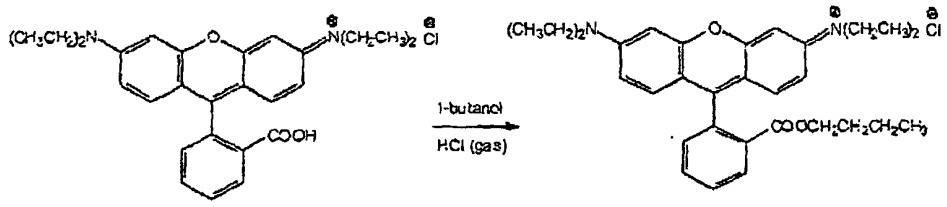
Other Clinical applications

In addition to using photosensitizers in the context of *in vitro* stem cell and immune cell purging for the prevention and treatment of graft-versus-host disease and other immunological disorders

Chemical Synthesis

All flash chromatography was done according to the method of Still et al. (Still W. C. et al. (1978) *J. Org. Chem.*, **43**: 2923). Thin-layer chromatography was conducted on silica Gel 60™ (HF-245, E. Merck) at a thickness of 0.20mm. Nuclear magnetic resonnance spectra were obtained with a Varian VXR 300™ (300MHz) instrument. Spectral data are reported in the following order: chemical shift (ppm), multiplicity, coupling constants, number of proton, assignment. Low resolution mass spectra using fast atom bombardment (FAB), were obtained on a Kratos MS-50 TA™ spectrometer. Ultraviolet spectra were obtained on a Varian DMS100™ spectrophotometer and data are presented as $\lambda/_{max}$.

1. Preparation of rhodamine B n-butylester



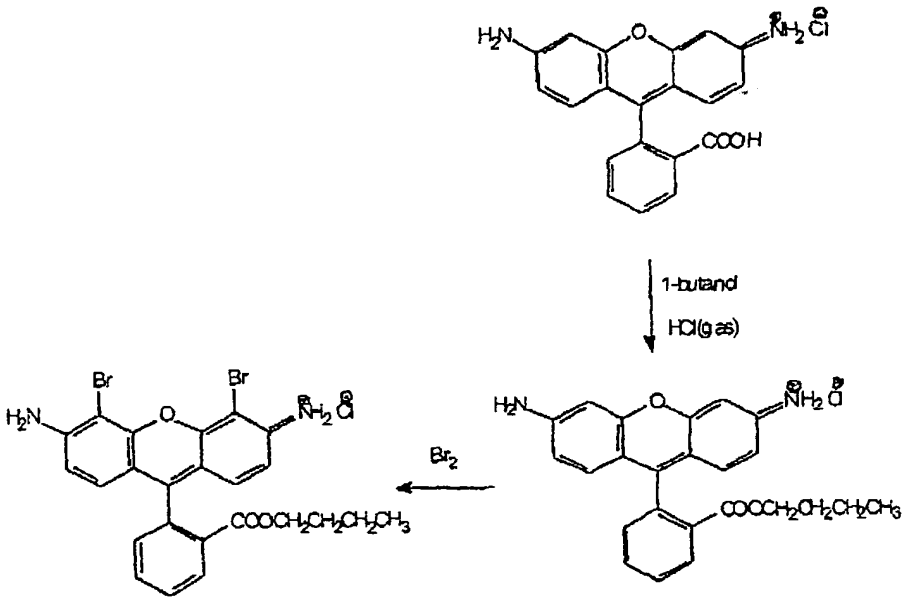
Rhodamine B hydrochloride (150 mg, 0.31 mmol) was dissolved in 1-butanol (5 ml). The reaction mixture was saturated with HCl (gas) and then stirred at 100 C for 15 hr. 1-Butanol was evaporated under reduced pressure. The crude oily residue was purified

by flash chromatography using CH_2Cl_2 (200 ml) and then CH_2Cl_2/CH_3OH (85:15) as eluant yielding 142 mg (0.27 mmol, 87% yield) of a dark red solid.

^1H NMR (Varian 300 MHz, Acetone, TMS)
d 8.31 (dd, J=1.4 and 7.8Hz, 1H); 7.86-7.94 (M, 2HO); 7.54 (dd, J=1.5 and 7.4Hz, 1H); 7.14-7.23 (M, 4H); 7.02 (d, J=2.2Hz, 2H); 3.97 (t, J=6.3Hz, 2H); 3.79 (q, J=7.1Hz, 8H); 1.32 (t, J=7.1Hz, 12H); 1.2-1.4 (M, 2H); 1.01 (h, J=7.5Hz, 2H); 0.75 (h, J=7.3Hz, 3H).

UV (methanol) $/_{max}$: 545nm

2. Preparation of dibromorhodamine n-butylester



2.1 Preparation of rhodamine n-butylester

Rhodamine 110 (14 mg, 0.038 mmol) was dissolved in 1-butanol (5 ml). The reaction mixture was saturated with HCl (gas) and then stirred at 100°C for 15 hr. The 1-Butanol was evaporated under reduced

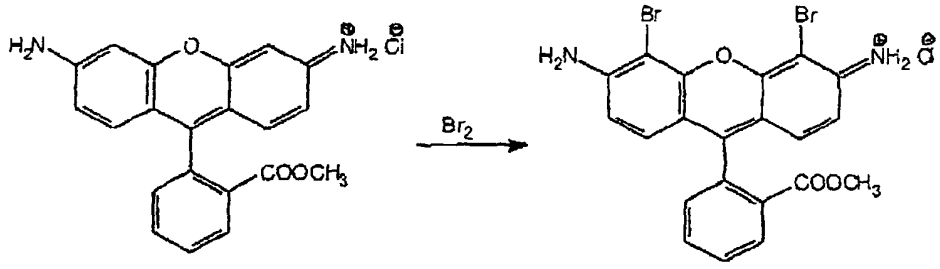
pressure. The crude oily residue was purified by flash chromatography using CH_2Cl_2/CH_3OH (85:15) as eluant yielding 14 mg (0.033 mmol, 87% yield) of a red solid.

2.2 Preparation of dibromorhodamine n-butylester

Rhodamine n-butylester (14 mg, 0.033 mmol) was dissolved in absolute ethanol (3 ml), then bromine (0.0036 ml, 0.070 mmol) was added. The mixture was stirred at room temperature for 1 hr. The solvent was evaporated and the crude reaction residue was purified by flash chromatography using CH_2Cl_2/CH_3OH (85:15) as eluant yielding 15.9 mg (0.027 Mol, 83% yield) of a red solid.

^1H NMR (Varian 300MHz, CD_3OD)
d 8.31 (dd, J=1.7 and 7.5Hz, 1H); 7.84 (M, 2H); 7.46 (dd, J=1.8 and 6.9Hz, 1H); 7.12 (d, J=9.2Hz, 2H); 7.03 (d, J=9.2Hz, 2H); 3.95 (t, J=6.2Hz, 2H); 1.22 (M, 2H); 0.93 (M, 2H); 0.75 (t, J=7.3Hz, 3H).
MS (LR,FAB) m/z: Calculated for C_{24}, $H_{21}N_2O_3Br_2$; 543
Observed: 543

3. Preparation of dibromorhodamine 123



pressure. The crude oily residue was purified by flash chromatography using CH_2Cl_2/CH_3OH (85:15) as eluant yielding 14 mg (0.033 mmol, 87% yield) of a red solid.

2.2 Preparation of dibromorhodamine n-butylester

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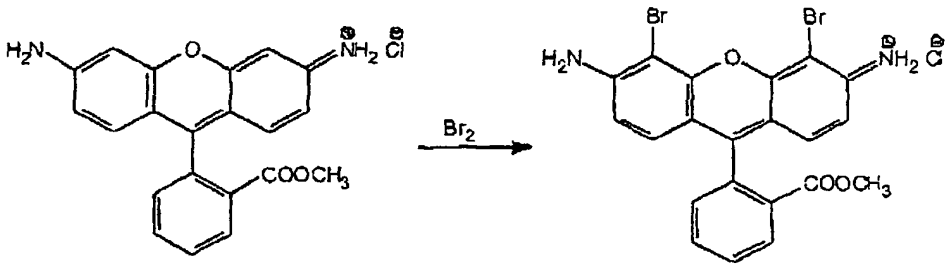
d 8.31 (dd, J=1.7 and 7.5Hz, 1H); 7.84 (M, 2H); 7.46 (dd, J=1.8 and 6.9Hz, 1H); 7.12 (d, J=9.2Hz, 2H); 7.03 (d, J=9.2Hz, 2H); 3.95 (t, J=6.2Hz, 2H); 1.22 (M, 2H); 0.93 (M, 2H); 0.75 (t, J=7.3Hz, 3H).
MS (LR,FAB) m/z: Calculated for C_{24}, $H_{21}N_2O_3Br_2$; 543
Observed: 543

3. Preparation of dibromorhodamine 123



To a solution of rhodamine 123 (25 mg, 0.066 mmol) in dry ethanol (1 ml), was added bromine (0.01 ml, 0.19 mmol) and the resulting mixture was stirred at room temperature for 0.5hr. Evaporation of solvent in vacuum provided the crude compound which was purified by flash chromatography using CH_2Cl_2/CH_3OH (85:15) as eluant yielding 27.0 mg (0.050 Mol, 77% yield) of a red solid.

^1H NMR (Varian 300MHz, CD_3OD)
d 8.34 (dd, J=1.7 and 7.5Hz, 1H); 7.85 (M, 2H); 7.46 (dd, J=1.7 and 7.2Hz, 1H); 7.10 (d, J=9.2Hz, 2H); 7.01 (d, J=9.2Hz, 2H); 3.64 (s, 3H). 8.3 (d, 1H, 9.1Hz, aromatic), 7.9 (m, 2H, aromatic), 7.45 (d, 1H, 9.1Hz, aromatic), 7.0, 7.2 (AB system, 4H, aromatic), 3.64 (s, 3H, OCH_3).

MS (LR, FAB) m/z: Calculated for C_{24}, $H_{21}N_2O_3Br_2$; 501

Observed: 501

UV (methanol)/$_{max}$: 510nm

Physical and photochemical properties

After synthesis, the purity of the preparation of the dyes was assessed by NMR analysis and was shown to be over 95%. Absorption and emission spectra were determined for each dye.

Cell lines

T cells represent the most important population of immune cells present in the peripheral blood. In order to demonstrate the efficacy of photodynamic therapy with TH9402 to eliminate activated T cells, we first evaluated its effect on a malignant T cell line. Phototoxicity was also evaluated in parallel against the chronic myelogenous leukemia cell line K562, that had been used in United States Patents Nos. 5,556,992

cand 5,773,460. The CEM T cell acute lymphoblastic leukemia cell line and K562 chronic myelogeneous leukemia cell line (Lozzio,B.B. and Lozzio,C.B. (1979) *Cancer Res.*, 3(6): 363-370) were obtained from the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852 USA) under the accession number CCL-119 and CCL-243. Cultures were maintained at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY)) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate, 100U/mL penicillin, and 100µg/mL streptomycin (Life Technologies, Inc.). Before each experiment, cell viability was assessed by trypan blue exclusion. CEM or K562 cells were admixed with normal irradiated mononuclear peripheral blood cells in a 1:1 ratio and then underwent photodynamic treatment. Before being mixed with CEM or K562 cells, normal PBMC received 25 Gy of radiation at 4 Gy/minute (^{137}Cs; Gamma Cell, Atomic Energy of Canada, Ottawa, ON).

Photodynamic treatment

Suspensions of cells were then incubated with 10 µM TH9402 for 40 minutes at 37°C. Cells were treated at 1 X 10^6 cells/mL in *ex vivo*-15 medium without phenol red (BioWhittaker, Walkersville, MD, USA). At the end of the incubation period, cells were spun down and the cell pellet resuspended in the *ex vivo* culture media in the absence of dye, supplemented with 10% AB serum. Cells were then placed in T-flasks (Corning, Cambridge, MA, USA) and incubated for 90 minutes at 37°C. Following this second incubation in medium without dye, cells were exposed to desired light energy, usually 5 joules/cm^2 using a previously described light delivery device (United States Patent 5,798,523). Light energy was delivered using a

fluorescent scanning lamp device with maximum wavelength around 512 nm.

Phototoxicity of 4,5-dibromorhodamine 123 (TH9402)

To assess the photochemotherapeutic potential and the *in vitro* phototoxicity of 4,5-dibromorhodamine 123 (TH9402), the T cell line CEM and the leukemic line K562 admixed with normal PBMC were incubated with TH9402 and exposed to 5 joules/cm^2 of light (as described above). After photodynamic treatment, cells were washed 3 times and plated in a limiting dilution assay (LDA) as described previously (Roy DC et al, *JNCI* 1996;88:1136-45). Briefly, each treatment sample was serially diluted from 5x10^5 to 0.5 cells per 100 µl in RPMI 1640 supplemented with 10% FBS. Then, 24 aliquots of each dilution were plated in flat bottom microculture plates (Nunclon, Nunc, Denmark). Cells were fed every 4 days and incubated at 37°C for 12-14 days. Growth at each serial dilution was assessed in an "all-or-nothing" (positive or negative) fashion under an inverted phase microscope. Frequency of clonogenic cells within the test population was estimated using chi-square minimization (Taswell C, *J.Immunol.* 1981;126:1614-19). As shown in Fig. 1, photodynamic therapy with TH9402 eliminated almost all CEM and K562 cells, with less than 0.1% of CEM and K562 escaping elimination by phototherapy in comparison to the media only sample. These results indicate high levels of elimination of malignant T cells, as was previously reported for leukemic K562 cells, and support efficacy of this procedure for the elimination of T cells. TH9402 was shown to be highly phototoxic; the elevated level of cytotoxic activity is believed to be a consequence of increased intersystem crossing of TH9402 to the triplet manifold via spin orbital-coupling induced by the heavy atoms.

T cell activation with PHA.

Normal PBMC were activated by incubation at 37°C for 48 hours in *Ex vivo*-15 medium (Biowhittaker, Walkersville. Md. U.S.A.) supplemented with 20% AB serum (Sigma), 1% pen-strep (Gibco), 2% glutamin (Gibco) and 10 µg of phytohemagglutinin-A (PHA-P). Cells were cultured in 25cm² flasks at a concentration of $3X10^6$ cells/ml. Following incubation, cells were washed and treated with the TH9402 photodynamic treatment as described above, and proliferative activity measured as described below.

Proliferation assay (mixed lymphocyte reaction)

To evaluate the residual proliferative potential of activated mononuclear cells after photodynamic therapy, peripheral blood mononuclear cells were placed in 96-well microtiter plates and were incubated with PBMC from various individuals (with known degrees of MHC incompatibility with treated cells). The latter cells were serially diluted in order to obtain effector (treated cells) to target ratios ranging from 2:1 to 1:8 ($4x10^4$ treated effector cells/well) and incubated at 37°C for 5 days. Eighteen hours prior to harvesting, 1µCi of ^{3}H-thymidine was added. Cells were harvested using a PHD cell harvester (Cambridge Technology, Boston, MA, USA). Radioactivity in the cell harvest was counted using a liquid scintillation counter (Beckman, Chicago, IL, USA).

Phototoxicity of 4,5-dibromorhodamine 123 against PHA activated cells

The phototoxicity of TH9402 against PHA-activated PB mononuclear cells was assessed after photodynamic treatment using 5 and 7.5 joules/cm² of light energy. After treatment, the cells were washed and evaluated for proliferative activity in a mixed

lymphocyte reaction, according to the protocol in the previous paragraph. In PHA-activated cells that did not receive photodynamic therapy (untreated), proliferation in mixed lymphocyte culture increased with the number of target cells. In contrast, when PHA-activated cells were treated with TH9402 using 5 and 7.5 J/cm^2 light energies, reactivity towards MHC incompatible cells was abrogated. This result indicates that photodynamic therapy of PHA-activated cells is a very potent inhibitor of immunoreactivity in these cells. Cell counts performed three days after the photodynamic treatment show a decrease by more than ninety percent (90%) of the treated cells in comparison to the medium control. These results indicate that the loss of proliferative activity in activated cells is most likely due to the elimination of effector cells.

Allogeneic T cell activation

Another appproach was used in this study to activate cells against specific target antigens. Mononuclear cells from subject A were incubated with irradiated mononuclear cells from subject B. In this one-way mixed lymphocyte culture, subjects A and B were unrelated and showed only partial human leukocyte antigen (HLA)matching with differences at two major histocompatibility complex (MHC) antigens. Briefly, 25X10^5 PBMC were incubated at 37°C for 4 days with 25X10^6 irradiated (25Gy) stimulating mononuclear cells in *Ex vivo*-15 medium (BioWhittaker) supplemented with 20% AB serum (Sigma), 1% pen-strep (Gibco), 2% glutamin (Gibco) and 50U/ml of IL-2 (ID lab). All cultures were performed in 75cm^2 flasks (Corning) in a

final volume of 25 ml. The unstimulated control was performed with $25X10^6$ irradiated autologous cells.

After this activation period, cells had photodynamic therapy with TH9402 as described above. Following treatment, cells were plated in a proliferation assay where targets consisted of PBMC from subject B and also from subject C (mismatched unrelated). As shown in Figure 4, when cells from subject A, activated against B, underwent TH9402 photodynamic therapy, they did not proliferate when reexposed to cells from B. However, when the same A cells were exposed to C cells, they had retained the capacity to proliferate. These results indicate that photodynamic therapy can specifically eliminate alloreactive cells, while sparing the alloreactive potential of unactivated cells. In addition, they demonstrate that it is possible to take advantage of this activation strategy to deplete immunoreactive populations against a desired antigen.

Cellular concentration of TH9402

TH9402 cellular content in resting and activated lymphocytes was evaluated by immunofluorescence, since the intensity of TH9402 (green) fluorescence correlates with the cellular content in TH9402. Briefly, 10^6 cells/ml, previously activated or not with PHA, were incubated in Ex vivo-15 medium supplemented with 2.5% human AB serum and 10µm TH9402 for 40 minutes. These cells were washed two times with *ex vivo* medium supplemented with 10% AB serum and cells analysed by flow cytometry 30, 50, 70, 90 and 110 min after the end of the TH9402

incorporation period. Resting lymphocytes rapidly lost TH9402 with approximately 50% (fifty percent) of cells demonstrating low TH9402 fluorescence 110 minutes after the end of the incorporation period. In addition, at all time-points evaluated, the intensity of TH9402 fluorescence was less for resting lymphocytes than for activated lymphocytes. Since cellular concentration of TH9402 correlates with the extent of cell elimination, the high concentration of TH9402 maintained in activated lymphocytes explains their sensitivity to photodynamic therapy. In contrast, the rapid efflux of TH9402 from resting lymphocytes should explain preservation of their proliferative activity.

In order to identify the mechanism responsible for the differential retention of TH9402 between activated and resting lymphocytes, cyclosporin-A was used to block the multidrug transporter (P-gp 170). These cells were incubated with 10μM TH9402 for 8 minutes, and washed with medium containing 1μg/ml cyclosporin-A or medium alone. TH9402 retention was subsequently evaluated by flow-cytometry (green fluorescence)(Figure 4). After 110 minutes from the end of TH9402 incorporation, fluorescence intensity was identical in activated cells treated or not treated with cyclosporin A. In contrast, cyclosporin A induced higher retention of TH9402 in resting lymphocytes, suggesting that a functional P-gp is involved in TH9402 dye efflux from resting lymphocytes and represents a major mechanism whereby these cells escape elimination by photodynamic therapy. The absence of such a pump in activated lymphocytes could

explain the high levels of phototoxicity observed in these cells.

Phototoxicity of 4,5-dibromorhodamine 110 n-butyl ester

To ascertain the photochemotherapeutic potential of 4,5-dibromorhodamine 110 n-butyl ester (DBBE), *in vitro* phototoxicity was evaluated in the K-562 cell line procedure described. The cells were incubated with increasing concentrations of DBBE and the cell viability was measured at different time points following photodynamic therapy. The results shown in Figs. 6A, 6B and 6C show that a dosage of 10 µg/ml of the dye and a brief exposure to 514.5 nm radiation from an argon ion laser at 0.5 J/cm^2 completely suppress cell viablity in less than 24 hours after irradiation.

Photo toxicity of Rhodamine B n-butyl ester

The photo toxicity *in vitro* of rhodamine B n-butyl ester (RBBE) was evaluated in the K-562 cell line procedure, in order to assess its photochemotherapeutic potential. Comparison was made to the induced phototoxicity of rhodamine 123 (123RH) and of rhodamine B butyl ester. Cell viability was evaluated 2 and 20 hours after photodynamic therapy. The results shown in Figs. 7A and 7B demonstrate that a dosage of 10 µg/ml of the dye and a photo exposure of 5 J/cm^2 from argon ion laser (514.5 nm) significantly suppress cell viablity of K562 cells in less than 20 hours after irradiation. Rhodamine 123 has no effect on cell viability, even at exposures of 10 J/cm^2. Phototoxicity of 4,5-dibromorhodamine 110 n-butyl ester and rhodamine B n-butyl ester were only assessed against the cell line K562. However, we

anticipate that their activity will be similar against T cells.

Phototoxicitity against hematopoietic progenitor cell cultures

It is observed that the photo treatment alone, at energy levels up to 10 J/cm2, or the pre-incubation of the cells at saturating concentrations of the dyes did not affect neither the establishment of the long term culture nor the formation in semi solid assays of cellular colonies issued from the multiplication and differentiation of committed progenitors present in the bone marrow (colony forming units-erythrocytes (CFU-E), blast forming units-erythrocytes (BFU-E), colony forming units-granulocytes, macrophages, (CFU-G-M)). However, as reported for rhodamine 123, the LTC (Long Term Culture) establisment is more sensitive to the dyes but the number of viable commited precursor and stem cells remains unaffected. Photodynamic therapy with rhodamine 123, rhodamine B n-butyl ester and 4,5-dibromorhodamine n-butyl ester minimally impaired the establishment of normal mouse long term culture of bone marrow and the formation of hematopoietic colonies in semi-solid assays. This is in agreement with results obtained previously in other laboratories using rhodamine 123.

Conventional approaches for the prevention and treatment of immunologic disorders such as immunosuppressive agents, radiotherapy and monoclonal antibody-based therapies are limited by their intrinsic toxicity and myelosuppressive effects. The introduction of strategies to eliminate T cells *in vitro* or *in vivo* has resulted in a decreased incidence of graft-versus-host disease after allogeneic stem cell transplantation, improved graft survival in solid organ transplantation and improved clinical conditions

for patients with immunologic disorders. However, T cell depletion is associated with an increased incidence of infections and malignancies or recurrence of malignant diseases, which have limited the use of T cell elimination strategies. These complications are primarily attributable to the non-specific elimination of a majority of T cells, which are responsible for the control of infection and anti-leukemia activity. To overcome these limitations and to expand the number of patients and age limit for intensive curative therapy, the potential benefit of selective *in vitro* elimination of immunoreactive cells prior to allogeneic stem cell transplantation has become widely acknowledged. Moreover, selective elimination of immunoreactive cells has the potential to be most useful in the context of donor lymphocyte infusion after transplantation, solid organ transplantation, and autoimmune disorders where the patient might benefit from the elimination of alloreactive or activated immune cell populations.

In an effort to develop new anti-neoplastic drugs that would allow selective destruction of alloreactive or activated immune cells, new dye molecules have been prepared and tested as possible new photosensitizers, useful for the photodynamic prevention and therapy of immunologic disorders. Three new photosensitizers of the pyrylium family were prepared and there is provided evidence for their potential use in the photodynamic treatment of immunologic disorders and also in the prevention and/or treatment of graft-versus-host disease.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Method of prevention of graft-versus-host disease in the context of allogeneic stem cell transplantation

1. Diagnosis and identification of immunological differences between donor and recipient, and graft-versus-host disease:

Allogeneic stem cell transplantation is performed for numerous neoplastic and non-neoplastic conditions. Hematological malignancies are comprised of leukemia, lymphoma, multiple myeloma, myelodysplastic syndromes, etc.; and non-hematological malignancies: aplastic anemia, congenital disorders, severe immunodeficiency syndromes, rhumatoid arthritis, scleroderma, lupus erythematosus, multiple sclerosis, and other immune disorders.

Graft-versus-host disease is a complication of allogeneic stem cell transplantation, where donor cells react against host cells, damaging target tissues (usually skin, liver, gut, lung, lacrymal or salivary glands, etc.). The diagnosis relies on several clinical and laboratory parameters, that are extensively reviewed in *Graft-vs.-Host Disease*, Ferrara JLM, Deeg HJ, Burakoff SJ eds, Marcel Dekker, New York, 1997.

GVHD develops against antigens present on recipient cells but not on donor cells. Immunological differences between donor and recipient could be present at the level of major histocompatibility antigens, minor histocompatibility antigens or tumor-associated antigens. Disparity will be established using one or more of the following procedures on blood or bone marrow cells:

a) HLA typing: conventional serologic typing or molecular to identify disparities between donor

and recipient in major histocompatibility complex class I and class II antigens; and

b) Mixed lymphocyte culture to identify differences in class II antigens; and

c) Minor histocompatibility antigens: although a few cytotoxic T cell lines are available and could be used to identify minor histocompatibility antigens, currently, these tests are only available for research purposes.

2. **Progenitor cell harvesting**

After diagnosis, bone marrow (BM) or peripheral blood (PB) or cord-blood derived hemopoietic stem cells from the donor will be harvested using previously described procedures for allogeneic progenitor cell transplantation (reviewed in *Bone Marrow Transplantation*, Forman SJ, Blume KG, Thomas ED eds, Blackwell Scientific Publications, Cambridge MA, USA, 1994). Donor hemopoietic stem cells collected for allografting will be immediately incubated with irradiated (25Gy) host mononuclear or other cells. Host cells admixed with donor cells are incubated in sterile dye free medium supplemented with 20% autologous serum and interleukin-2 for 2 days. This procedure elicits donor cell alloreactivity towards the host, and the cell graft subsequently undergoes photodynamic treatment *ex vivo* as described below.

3. **Selective *in vitro* purging of immunoreactive cells**

Ex vivo treatment will consist of short-term incubation of previously activated BM or PB stem cells with one or several of the selected photoactive compounds. Duration of incubation, cell concentration and drug molarity will be determined for each patient using an aliquot of the harvested cell population.

Excess of dyes will be removed by cell washes with sterile dye free medium supplemented with 2% autologous serum. Cells will next be exposed to radiant energy of sufficient intensities to effect photodynamic purging of leukemia cells. Efficacy of the photodynamic purging procedure will be verified on an aliquot of the treated cell population, before cryopreservation and/or re-infusion to the patient is performed. Until re-infusion to the patient, the cells will be cryopreserved in 10% dimethylsulfoxyde (DMSO) - 90% autologous serum medium, at -196°C in the vapor phase of liquid nitrogen.

4. Systemic treatment of patients

Following stem cell harvest, the patient will be submitted to dose-intensive chemotherapy and/or irradiation when indicated.

5. Allogeneic stem cell transplantation

Following appropriate treatment of the patient by high-dose chemotherapy and/or irradiation and at the appropriate clinical moment, cryopreserved marrow or peripheral blood or cord blood stem cells will be rapidly thawed and returned to the patient.

EXAMPLE II

Method of treatment of graft-versus-host disease and autoimmune diseases

1. Diagnostic procedures

Diagnosis of graft-versus-host disease or immunoreactive disorders will be established using conventional clinical, biochemical and/or histopathological examination of the blood or appropriate tissues. Diagnostic and predictive features of GVHD are reviewed in *Graft-vs.-Host*

Disease, Ferrara JLM, Deeg HJ, Burakoff SJ eds, Marcel Dekker, New York, 1997.

2. Harvesting of peripheral blood cells

After diagnosis of severe GVHD, autoimmune or immunoreactive disorder, peripheral blood (PB) mononuclear cells will be harvested using previously described or similar leukopheresis procedures (reviewed in *Bone Marrow Transplantation*, Forman SJ, Blume KG, Thomas ED eds, Blackwell Scientific Publications, Cambridge MA, USA, 1994). Patient's peripheral blood mononuclear cells collected will be treated immediately *ex vivo* as described below.

3. *In vitro* elimination of cells mediating GVHD

Ex vivo treatment will consist of short-term incubation of PB stem cells with one or several of the selected photoactive compounds. Duration of incubation, cell concentration and drug molarity will be determined for each patient using an aliquot of the harvested cell population. Excess of dyes will be removed by cell washes in sterile dye free medium supplemented with 2% autologous serum. Cells will next be exposed to radiant energy of sufficient intensities to effect photodynamic purging of activated cells which mediate GVHD.

4. Administration of photodynamically treated cells to patients

Leukopheresed cells that are photodynamically treated will be reinfused into the patient. This approach will enable the elimination of a large number of circulating activated lymphocytes and other cells involved in GVHD. In addition, cells spared by the photodynamic treatment are unactivated and their

reinfusion into the patient may help restore normal immunologic equilibrium.

EXAMPLE III

Method of treatment of immunologic disorders

1. Diagnostic procedures

Diagnosis of autoimmune disorders will be established using conventional clinical, biochemical and/or histopathological examination of the blood or appropriate tissues. Severe autoimmune diseases are amenable to autologous transplantation (reviewed in Sullivan KM et al., *Am. Soc. Hematol.*, Educ.Program Book,1998:198-214).

2. Harvesting of hematopoietic stem cells

After diagnosis, bone marrow (BM),peripheral blood (PB) or cord blood (CB) mononuclear cells will be harvested using previously described procedures for the autologous marrow transplantation in cancer therapy (reviewed in *Bone Marrow Transplantation*, Forman SJ, Blume KG, Thomas ED eds, Blackwell Scientific Publications, Cambridge MA, USA, 1994). Patient's hemopoietic stem cells collected for autograft will be treated immediately *ex vivo* as described below.

3. *In vitro* elimination of cells mediating autoimmune disorders

Ex vivo treatment will consist of short-term incubation of BM or PB stem cells with one or several of the selected photoactive compounds. Duration of incubation, cell concentration and drug molarity will be determined for each patient using an aliquot of the harvested cell population. Excess of dyes will be removed by cell washes in sterile dye free medium supplemented with 2% autologous serum. Cells will

next be exposed to radiant energy of sufficient intensities to effect photodynamic purging of immunoreactive cells which mediate the immunologic disorder.

4. Administration of photodynamically treated cells to patients

Hematopoietic stem cells that are photodynamically treated will be stored (frozen or kept in culture). This approach will enable the elimination of a large number of activated lymphocytes and other cells involved in the immunologic disorder. In addition, cells spared by the photodynamic treatment are unactivated and their reinfusion may help restore normal immunologic equilibrium. Following stem cell harvest, patient will be either treated with conventional regimens until autografting is clinically indicated or immediately submitted to dose-intensive chemotherapy and total body irradiation where indicated.

5. Autologous stem cell transplantation

Following high-dose chemotherapy and irradiation cryopreserved marrow or peripheral blood stem cells will be rapidly thawed and infused to the patient.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set

forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A photoactivable pharmaceutical composition for the selective destruction and/or inactivation of immunologically reactive cells without affecting the normal cells and without causing systemic toxicity for the patient, said composition comprising at least one photoactivable rhodamine derivative selected from the group consisting of 4,5-dibromorhodamine 123 (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid methyl ester hydrochloride); 4, 5-dibromorhodamine 123 (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid ethyl ester hydrochloride); 4, 5-dibromorhodamine 123 (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid octhyl ester hydrochloride); 4,5-dibromorhodamine 110 n-butyl ester (2-(4,5-dibromo-6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid n-butyl ester hydrochloride); Rhodamine B n-butyl ester (2-(6-ethyl amino-3-ethyl imino-3H-xanthen-9-yl)-benzoic acid n-butyl ester hydrochloride); and photoactivable derivatives thereof; in association with a pharmaceutically acceptable carrier; whereby photoactivation of said derivatives induces cell killing while unactivated derivatives are substantially non-toxic to cells.

2. Use of the photoactivable derivatives of claim 1 for the photodynamic treatment for the selective destruction and/or inactivation of immunologically reactive cells without affecting the normal cells and without causing systemic toxicity for the patient, wherein appropriate intracellular levels of said derivatives are achieved and irradiation of a suitable wavelength and intensity is applied.

3. A method of prevention of graft-versus-host disease associated with allogeneic stem cell transplantation in a patient, which comprises the steps of:

a) activating lymphocytes from a donor by mixing donor cells with host cells for a time sufficient for a period of time sufficient for an immune reaction to occur;

b) substantially eliminating the activated lymphocytes of step a) with photodynamic therapy using a therapeutic amount of a photoactivable composition of claim 1 under irradiation of a suitable wavelength; and

c) performing allogenic stem cell transplantation using the treated mix of step b).

4. A method for the treatment of immunologic disorder in a patient, which comprises the steps of:

a) harvesting said patient's hematopoietic cells;

b) *ex vivo* treating of the hematopoietic cells of step a) by photodynamic therapy using a therapeutic amount of a photoactivable composition of claim 1 under irradiation of a suitable wavelength; and

c) performing graft infusion or autograft transplantation using the treated hematopoietic cells of step b).

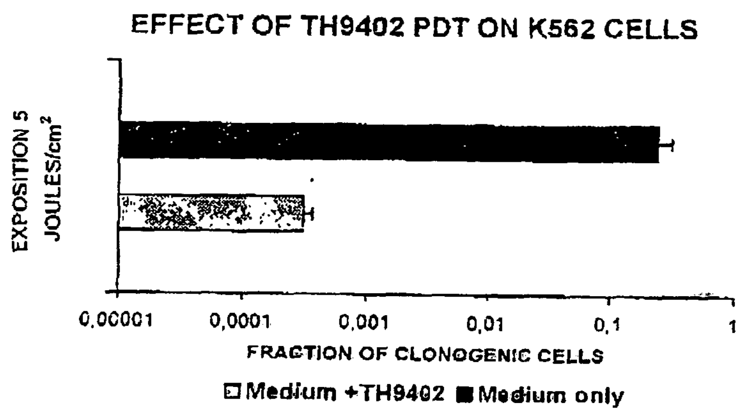
5. The method of claim 4, wherein said immunologic disorder is selected from the group consisting of conditions in which self cells or donor cells react against host tissues or foreign targets, such as garft-

versus-host disease, graft rejection, autoimmune disorders and T-cell mediated immunoallergies.

6. The method of claim 4, wherein said hematopoietic cells is selected from the group consisting of bone marrow, peripheral blood, and cord blood mononuclear cells.

ABSTRACT OF THE INVENTION

The present invention relates to the use of the photoactivable derivatives for the photodynamic treatment for the selective destruction and/or inactivation of immunologically reactive cells without affecting the normal cells and without causing systemic toxicity for the patient, wherein appropriate intracellular levels of said derivatives are achieved and irradiation of a suitable wavelength and intensity is applied.


EFFECT OF TH9402 PDT ON K562 CELLS
EXPOSITION 5 JOULES/cm²
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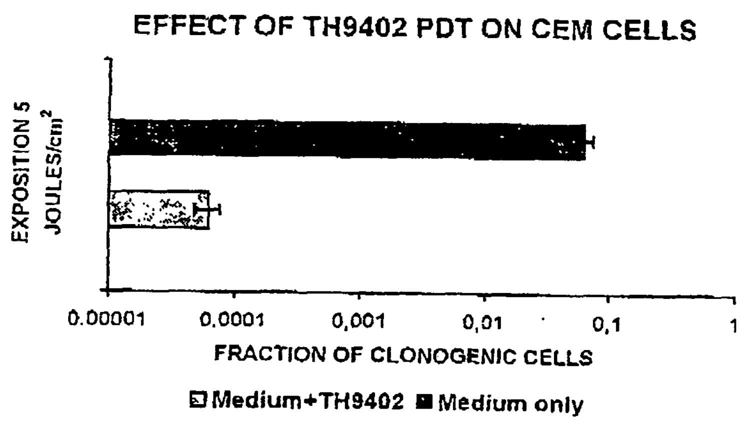
0,0001
0,001
0,01
0,1
1
FRACTION OF CLONOGENIC CELLS
Medium +TH9402
Medium only


EFFECT OF TH9402 PDT ON CEM CELLS
EXPOSITION 5 JOULES/cm²
0.00001
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0,001
0,01
0,1
1
FRACTION OF CLONOGENIC CELLS
Medium+TH9402
Medium only

Fig. 1

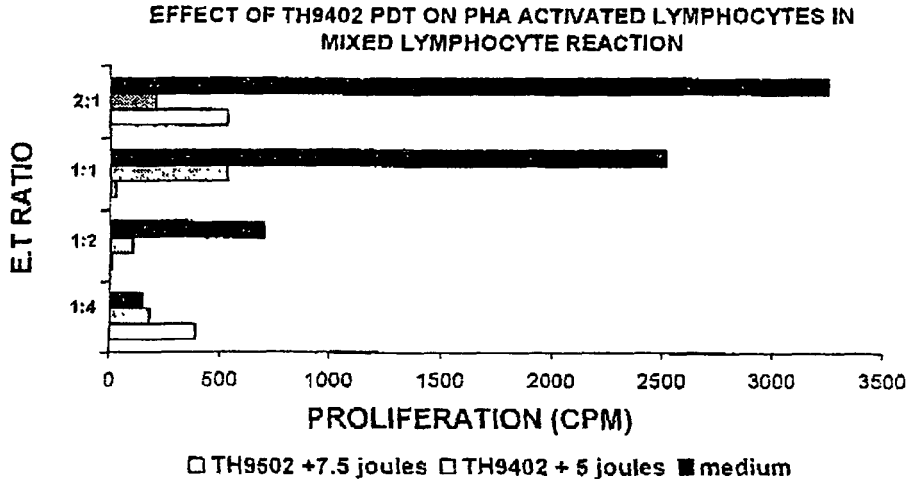

Fig. 2

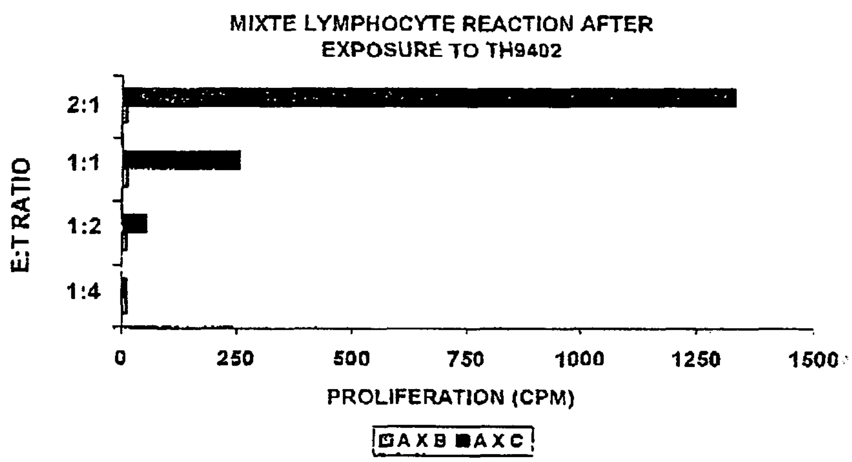
MIXTE LYMPHOCYTE REACTION AFTER
EXPOSURE TO TH9402
E:T RATIO
2:1
1:1
1:2
1:4
0
250
500
750
1000
1250
1500
PROLIFERATION (CPM)
A X B
A X C

Fig. 3

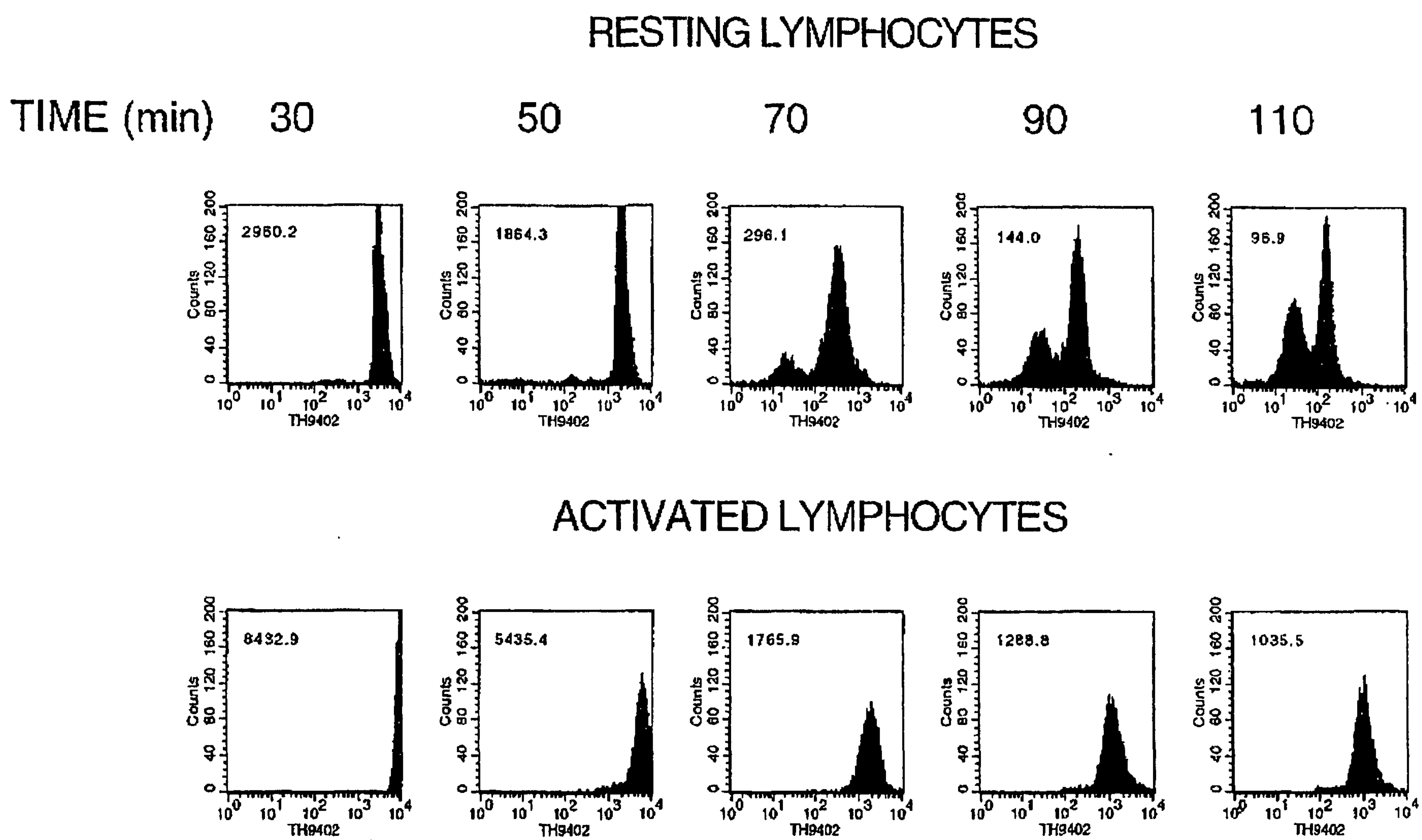
RESTING LYMPHOCYTES
TIME (min)
30
50
70
90
110
2960.2
1864.3
296.1
144.0
96.9
ACTIVATED LYMPHOCYTES
8432.9
5435.4
1765.9
1288.8
1035.5
Counts
TH9402

Fig. 4

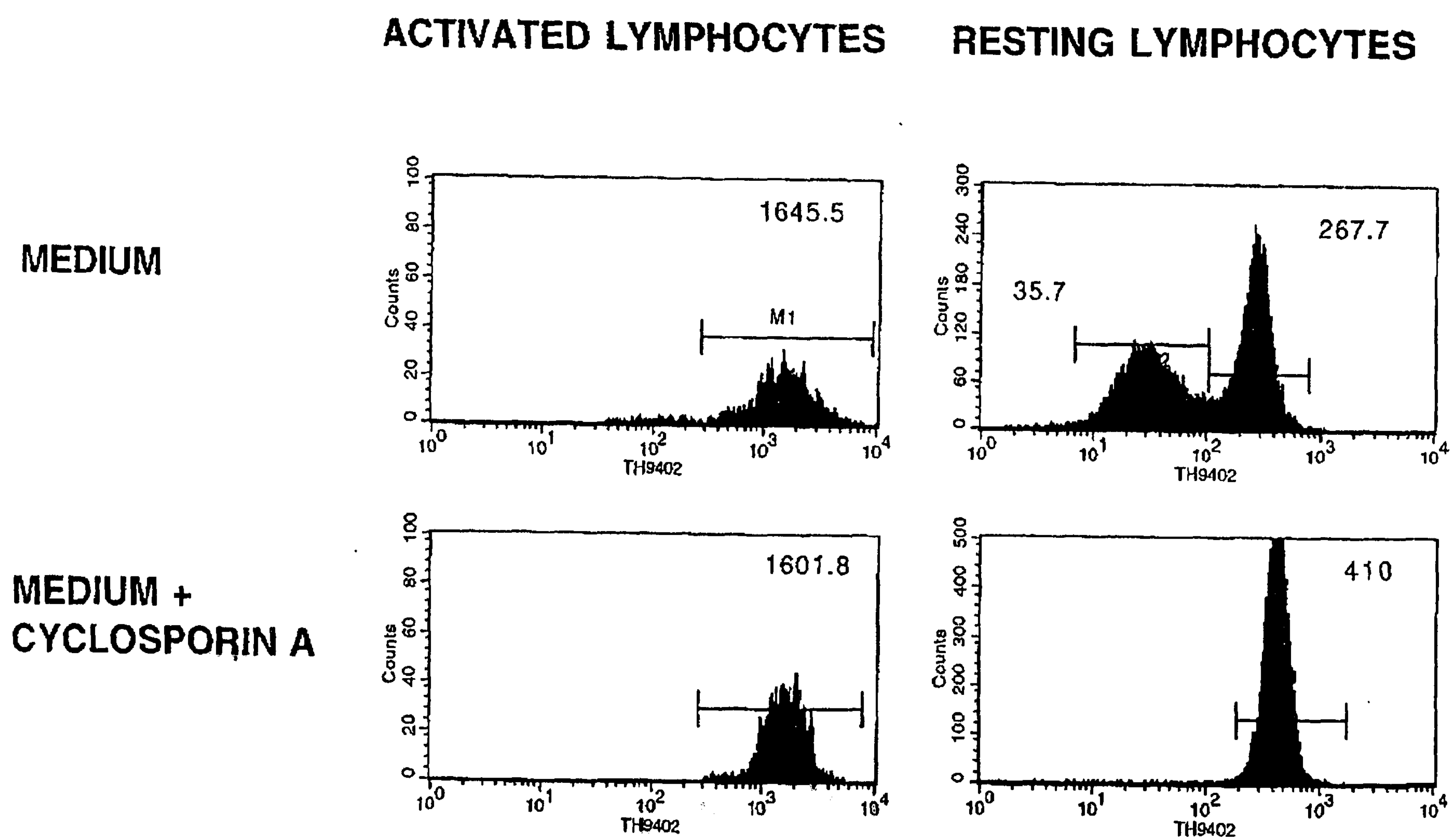
ACTIVATED LYMPHOCYTES
RESTING LYMPHOCYTES
MEDIUM
MEDIUM +
CYCLOSPORIN A
1645.5
M1
267.7
35.7
1601.8
410
Counts
TH9402

Fig. 5

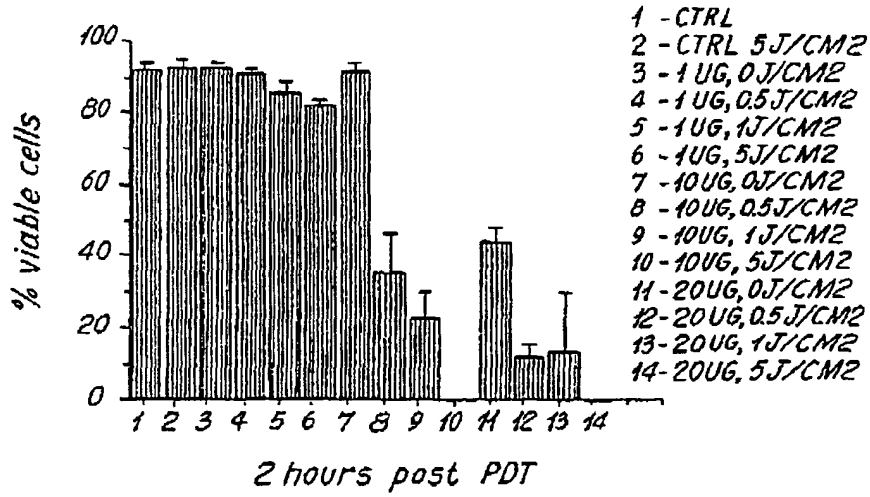

Fig. 6A

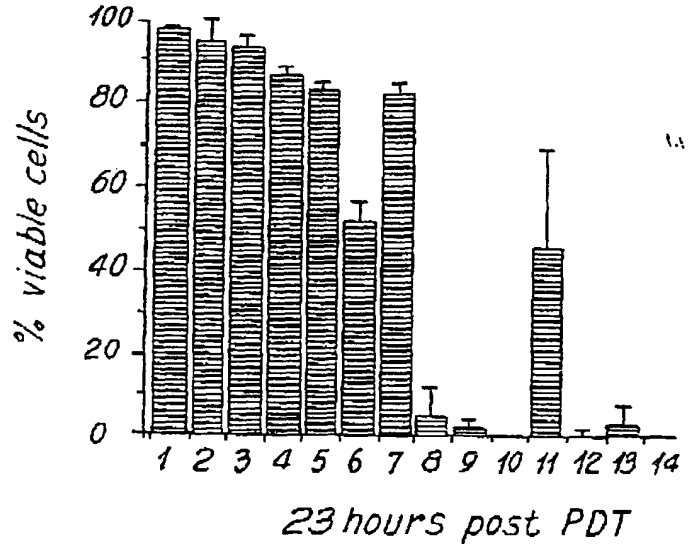
100
80
60
40
20
0
% viable cells
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23 hours post PDT

Fig. 6B

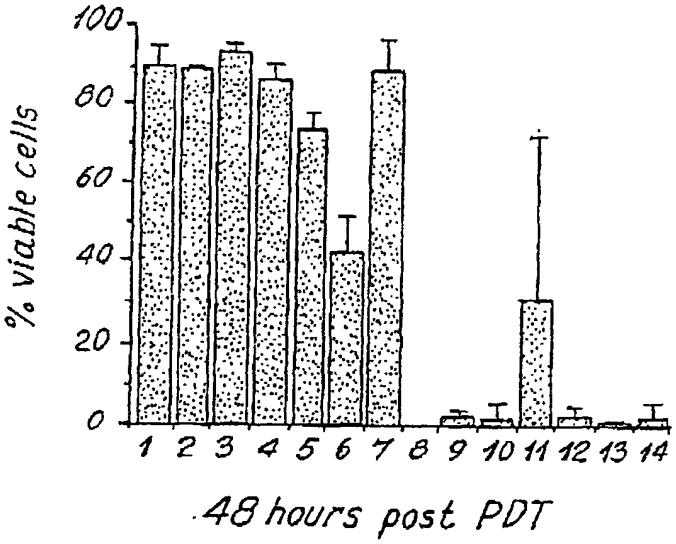
100
80
60
40
20
0
% viable cells
1 2 3 4 5 6 7 8 9 10 11 12 13 14
48 hours post PDT

Fig. 6C

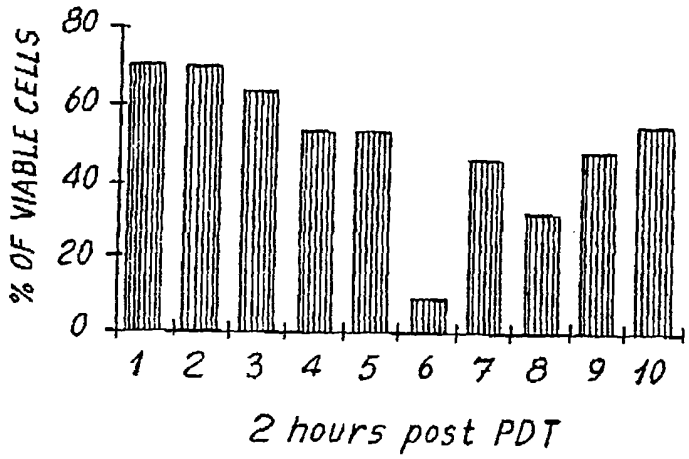

Fig. 7A

1-CONTROL, 0J/CM2
2-CONTROL, 5J/CM2
3-123RH, 0J/CM2
4-123RH, 5J/CM2
5-DBR+RBBE, 0J/CM2
6-DBR+RBBE, 5J/CM2
7-RBBE, 0J/CM2
8-RBBE, 5J/CM2
9-RBOE, 0J/CM2
10-RBOE, 5J/CM2

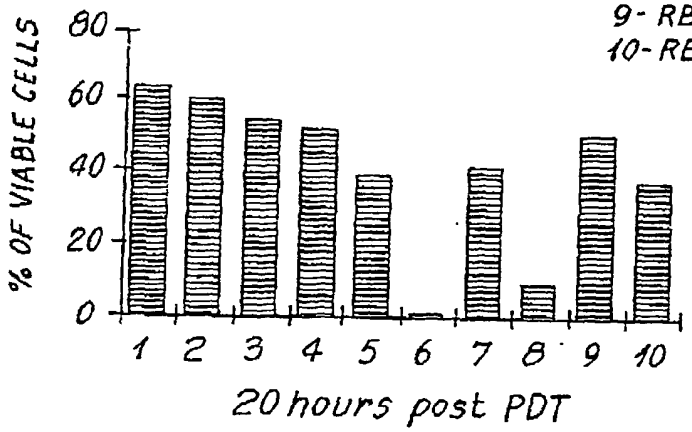

Fig. 7B